

REMARKS

Favorable reconsideration is respectfully requested in view of the following remarks.

I. CLAIM STATUS & AMENDMENTS

Claims 1-9 were pending in this application when last examined.

Claims 2, 3, 5, 6, 8 and 9 have been examined on the merits, and stand rejected.

Claims 1, 4 and 7 are withdrawn as non-elected subject matter.

II. FOREIGN PRIORITY CLAIM

As requested in the last response, kindly acknowledge the Applicants' claim for foreign priority under 35 U.S.C. § 119(a)-(d) or (f). The priority document was submitted to the PTO by the International Bureau as evidenced by item 1 in the Official Communication dated September 10, 2001.

III. UTILITY & ENABLEMENT REJECTIONS

Claims 2, 3, 5, 6, 8 and 9 remain rejected under 35 U.S.C. § 101, as lacking utility, and under 35 U.S.C. § 112, first paragraph, on the basis that specification lacks an enabling disclosure for how to use the claimed invention. See page 2 of the Advisory Action and pages 2-5 of the final Office Action.

These rejections are respectfully traversed for the reasons set forth in section III on pages 2-6 of the after final response filed December 23, 2004 and on pages 6-9 of the response filed March 23, 2004 and for the following reasons.

On page 2 of the Advisory Action, it is argued that the biological activity of the sequence of the instant invention has not been established. It is also asserted that the specific DNA targets are not disclosed or known such that the assertion that the sequence may treat or diagnose unspecified diseases lacks specific and substantial utility.

It is again respectfully submitted that the disclosed uses for the claimed polynucleotide encoding a novel human nuclear protein and the polypeptide itself are substantial and credible.

The mere fact that Chen discloses proteins with WW domains having varied functions does not negate the teachings in the specification regarding the biological activity of the polypeptide of the instant invention.

Again, the specification establishes that the human nuclear protein encoded by the claimed polynucleotide contains a WW domain (Example (i), page 10, lines 20-23) and that the protein exists in human cell nuclei (Example (vii), page 13, line 15 to page 14, line 9). The specification indicates that the claimed protein is involved in the signal transduction, as well as in ubiquitin-protein ligase in the protein degradation system and in a transcription activator. See page 2, lines 5-20 and page 10, lines 20-23. The Applicants also found that the protein encoded by the claimed polynucleotide binds the c-terminal domain (CTD) of RNA polymerase II and is involved in mRNA synthesis (Example (viii), page 14, lines 11-28).

The invention is useful to assess mRNA synthesis given its disclosed binding capacity to the C-terminal domain of RNA polymerase II. See page 7, lines 3-5 of the disclosure. It is noted that the specification also discloses antibodies prepared from the protein encoded by the claimed polynucleotide. Such antibodies would also be useful as tools to monitor signal transduction, transcription and mRNA synthesis in healthy and/or diseased cells.

Based on such teachings, it is respectfully submitted that one of skill in the art would reasonably recognize that the claimed polynucleotide sequence (SEQ ID NO: 2) and the human nuclear protein it encodes (SEQ ID NO: 1) have "real world" value as markers to assess and/or control signal transduction, transcription, and mRNA synthesis in healthy and/or diseased cells, and/or as diagnostic agents and medicaments for diseases involving signal transduction, transcription and mRNA synthesis, such as cancer such as cancer, Huntington's disease and Alzheimer's disease.

Furthermore, the PubMed search for "WW domains" attached to the prior response found around 91 articles published prior to the priority date of the instant invention. It is respectfully submitted that such results are evidence that the significance and utility of WW domain proteins have been well recognized by those in the art. For instance, among these references, it was

repeatedly pointed out that WW domain proteins play an important role in “intracellular signal transduction.”

In addition, it has been reported that WW domains relate to Huntington’s disease (Faber et al., “Huntingtin interacts with a family of WW domain proteins,” Human Mol. Genet., vol. 7, no. 9, pp. 1463-1474 (1998), a copy of which is enclosed). It has also been reported that: (1) WW domain proteins bound to phosphorylated CTD relate to Alzheimer’s disease (Sudol et al., “Function of WW domains in the nucleus,” FEBS Lett., vol. 490, no. 3, pp. 190-195 (2001) (Abstract enclosed); and (2) WW domain protein binding to phosphorylated proteins is involved in cancer and Alzheimer’s disease (Lu, “Pinning down cell signaling, cancer and Alzheimer’s disease,” Trends Biochem. Sci., vol. 29, no. 4, pp. 200-209 (2004) (Abstract enclosed)). Although Sudol and Lu were published after the priority date of the instant application, it is respectfully submitted that they further confirm the utility of the instant invention.

Also, it has been established that PCIF1 protein, which is the same 407 amino acid protein of the instant invention, controls RNA synthesis by adjusting the activity of RNA polymerase II (Fan et al., “PCIF1, a novel human WW domain-containing protein, interacts with the phosphorylated RNA polymerase II,” Biochem. Biophys. Res. Commun., vol. 301, no. 2, pp. 378-385 (2003) (Abstract enclosed). The significance of RNA synthesis for various diseases, such as cancer and neural disorders (Huntington’s disease and Alzheimer’s disease) were well known in the art.

Based on such findings, it is respectfully submitted that one of skill in the art, upon reading the disclosure and in view of the state of the art, would reasonably believe that the claimed polynucleotide sequence and the human nuclear protein it encodes are useful to assess signal transduction, transcription and mRNA synthesis in healthy and/or diseased cells, and/or as diagnostic agents and medicaments for diseases involving signal transduction, transcription and mRNA synthesis, such as cancer, Huntington’s disease and Alzheimer’s disease.

On page 2 of the Advisory Action, it is further argued that Applicants’ reliance on homology data which discloses a WW domain the polypeptide of the instant invention is unpersuasive, because (1) homology does not necessarily correlate to function; and (2) WW

domains are found in proteins with varied functions such that mere presence of a WW domain does not correlate to biological activity. Chen et al., J. Biol. Chem., vol. 272, no. 27, pp. 17070-7 (1990) is relied upon as showing the diversity of proteins having WW domains with varied functions.

It is respectfully submitted that such argument does not negate the teachings in the specification regarding the biological activity of the polypeptide of the instant invention for the reasons discussed above. Such argument does not diminish the use of the claimed invention to assess mRNA synthesis or signal transduction, which are two very important intracellular functions for the general cellular activities regardless of a disease condition or an ultimate function of the protein. In this regard, such protein could still be used to assess mRNA synthesis and signal transduction to monitor cellular development and activities. Moreover, as discussed above, the art has recognized the importance of the polypeptide of the instant invention to diseases, such as cancer, Huntington's disease and Alzheimer's disease. Clearly, such uses are credible and have real world application.

Thus, in view of the above, the utility and enablement rejections under 35 U.S.C. § 101 and under 35 U.S.C. § 112, first paragraph, are untenable and should be withdrawn.

Attorney Docket No.: 2001-1023A
Application No.: 09/889,722
July 25, 2005

CONCLUSION

In view of the foregoing amendments and remarks, the present application is in condition for allowance and early notice to that effect is hereby requested.

If the Examiner has any comments or proposals for expediting prosecution, please contact the undersigned attorney at the telephone number below.

Respectfully submitted,

Seishi KATO et al.

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July 25, 2005

Attorney Docket No.: 2001-1023A
Application No.: 09/889,722
July 25, 2005

ATTACHMENTS:

1. Fan Sakuraba et al., "PCIF1, a novel human WW domain-containing protein, interacts with the phosphorylated RNA polymerase II", Biochem Biophys Res Commun., Vol. 301, No. 2, pp. 378-385, (2003).
2. M. Sudol et al., "Functions of WW domains in the nucleus", FEBS Lett., Vol. 490, No. 3, pp. 190-195, (2001).
3. K.P. Lu, "Pinning down cell signaling, cancer and Alzheimer's disease", Trends in Biochem Sci., Vol. 29, No. 4, pp. 200-209, (2004).
4. Peter Faber et al., "Huntingtin interacts with a family of WW domain proteins", Human Molecular Genetics, Vol. 7, No. 9, pp. 1463-1474, (1998).



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1: Biochem Biophys Res Commun. 2003 Feb 7;301(2):378-
85.

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PCIF1, a novel human WW domain-containing protein, interacts with the phosphorylated RNA polymerase II.

Fan H, Sakuraba K, Komuro A, Kato S, Harada F, Hirose Y.

Division of Cell Biology, Cancer Research Institute, Kanazawa University,
13-1, Takara-machi, Kanazawa, Ishikawa 920-0934, Japan.

Phosphorylation of the carboxy-terminal domain (CTD) of RNA polymerase II (RNAP II) largest subunit has an important role in transcription elongation and in coupling transcription to pre-mRNA processing. To identify proteins that can directly bind to the phosphorylated CTD, we screened a human cDNA expression library using 32P-labeled CTD as a probe. Here we report the cloning and characterization of a novel human WW domain-containing protein, PCIF1 (phosphorylated CTD interacting factor 1). PCIF1 is composed of 704 amino acids. The WW domain of PCIF1 can directly and preferentially bind to the phosphorylated CTD compared to the unphosphorylated CTD. PCIF1 binds to the hyperphosphorylated RNAP II (RNAP IIO) in vitro and in vivo. Double immunofluorescence labeling in HeLa cells demonstrated that PCIF1 and endogenous RNAP IIO are co-localized in the cell nucleus. Thus, PCIF1 may play a role in mRNA synthesis by modulating RNAP IIO activity.

PMID: 12565871 [PubMed - indexed for MEDLINE]

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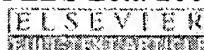
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1: FEBS Lett. 2001 Feb 16;490(3):190-5.

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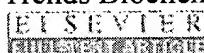
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1: Trends Biochem Sci. 2004 Apr;29(4):200-9.

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Pinning down cell signaling, cancer and Alzheimer's disease.

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Protein phosphorylation on certain serine or threonine residues preceding proline (Ser/Thr-Pro) is a pivotal signaling mechanism in diverse cellular processes and its deregulation can lead to human disease. However, little is known about how these phosphorylation events actually control cell signaling. Pin1 is a highly conserved enzyme that isomerizes only the phosphorylated Ser/Thr-Pro bonds in certain proteins, thereby inducing conformational changes. Recent results indicate that such conformational changes following phosphorylation are a novel signaling mechanism pivotal in regulating many cellular functions. This mechanism also offers new insights into the pathogenesis and treatment of human disease, most notably cancer and Alzheimer's disease. Thus, Pin1 plays a key role in linking signal transduction to the pathogenesis of cancer and Alzheimer's disease - two major age-related diseases.

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Huntingtin interacts with a family of WW domain proteins

Peter W. Faber, Glenn T. Barnes, Jayalakshmi Srinidhi, Jianmin Chen,
James F. Gusella and Marcy E. MacDonald*

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Received May 11, 1998; Revised and Accepted July 2, 1998

The hallmark neuropathology of Huntington's disease (HD) is due to elongation of a polyglutamine segment in huntingtin, a novel ~350 kDa protein of unknown function. We used a yeast two-hybrid interactor screen to identify proteins whose association with huntingtin might be altered in the pathogenic process. Surprisingly, no interactors were found with internal and C-terminal segments of huntingtin. In contrast, huntingtin's N-terminus detected 13 distinct proteins, seven novel and six reported previously. Among these, we identified a major interactor class, comprising three distinct WW domain proteins, HYPA, HYPB and HYPC, that bind normal and mutant huntingtin in extracts of HD lymphoblastoid cells. This interaction is mediated by huntingtin's proline-rich region and is enhanced by lengthening the adjacent glutamine tract. Although HYPB and HYPC are novel, HYPA is human FBP-11, a protein implicated in spliceosome function. The emergence of this class of proteins as huntingtin partners argues that a WW domain-mediated process, such as non-receptor signaling, protein degradation or pre-mRNA splicing, may participate in HD pathogenesis.

INTRODUCTION

Huntington's disease (HD) is a dominantly inherited neurodegenerative disorder featuring progressively worsening chorea, psychiatric disturbances and cognitive impairment due to neuronal cell loss in the basal ganglia and the cerebral cortex (1,2). It is caused by expansion of a polymorphic CAG trinucleotide repeat (6–34 units) (3) that encodes a glutamine tract in huntingtin, a ~350 kDa protein of unknown function that is essential for normal embryonic development (4–6) and neurogenesis (7). The length of the CAG repeat on disease chromosomes (35 to >100 units) is correlated with age at neurologic onset, such that the majority of cases show adult onset due to ~42–48 CAGs, while the longest arrays (>60 units) cause juvenile onset HD (3,8).

The HD mutation confers a new property on huntingtin that is specifically deleterious to target neurons and is likely to be subtle, given the late age of onset and long time course of the disorder

(9–11). The glutamine tract extended by the HD defect is adjacent to a mildly polymorphic proline-rich segment (12) in huntingtin's N-terminus and alters the physical properties of the mutant protein as evidenced by decreased mobility on SDS-PAGE (13–17) and increased reactivity with specific monoclonal antibody reagents (7,18). Aberrant behavior of mutant huntingtin is also reflected by the formation of cytoplasmic and nuclear inclusions in HD brain (19), although it is not yet clear whether these are a cause or a consequence of the pathogenic process (20). The distinct physical properties of mutant huntingtin suggest that HD pathology may be triggered by an altered interaction with another protein, either a change in the association with a normal binding partner or an abnormal binding with a novel partner.

To identify candidates for involvement in such an interaction, we have used yeast two-hybrid cDNA expression library screening (21). We have identified a number of interactors for huntingtin's N-terminus that suggest a variety of cellular processes to be explored. Most notably, a major class of proteins represented by three independent members implicates the WW domain, an ancient protein motif involved in non-receptor signaling, channel function, protein processing and pre-mRNA splicing, as a potential participant in huntingtin's inherent function and the pathogenesis of HD (22).

RESULTS

Isolation of huntingtin yeast partners (HYPs)

It has been proposed that altered huntingtin–protein interaction(s) are involved in HD pathogenesis (9–11,20). Consequently, we have surveyed this ~350 kDa protein using distinct yeast two-hybrid systems featuring *LexA* (23) or *GAL4* (24) bipartite DNA binding–activation domains. We generated six bait clones that express part of huntingtin (Fig. 1) as a fusion protein: three evolutionarily conserved internal segments containing HEAT motifs (25) (pGBT9-HD530–1050, pLexA-HD1434–1721, pGBT9-HD1434–1721), the C-terminus (pGBT9-HD2226–3144) and the N-terminus with 58 and 62 glutamines (pGBT9-HD1–550Q58, pLexA-HD1–425Q62). We used these baits to screen two fetal brain (*LexA* and *GAL4*) and a testis (*GAL4*) cDNA library and subsequently to validate the auxotrophy and β-galactosidase phenotypes of candidate interactor clones based on stringent selection criteria (see Materials and Methods). A summary of the results is given in Table 1.

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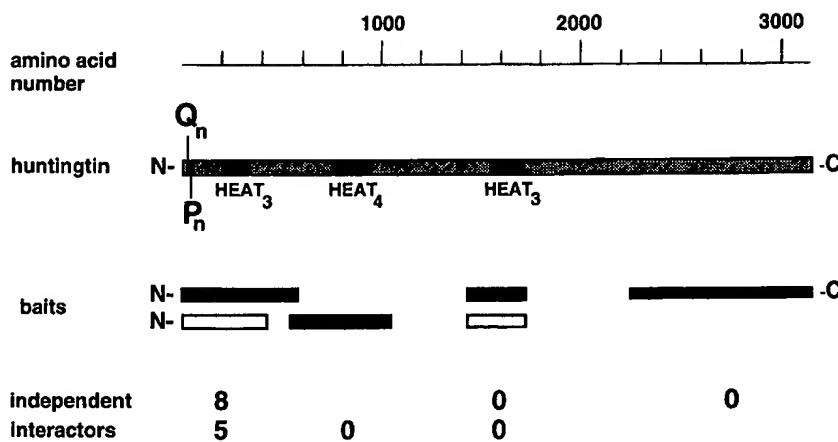


Figure 1. Huntingtin yeast two-hybrid strategy. The position of huntingtin's variable polyglutamine (Q_n) and proline-rich (P_n) tracts and segments with either three or four HEAT repeats (HEAT) are depicted on a schematic diagram of the full-length, 3144 amino acid, protein (shaded bar) (accession no. L12392). The line below indicates the relative locations of the huntingtin fusion protein fragments comprising bait clones, pLexA-HD1-425Q62, pLexA-HD1434-1721 (open boxes), pGBT9-HD1-550 Q58, pGBT9-HD530-1050, pGBT9-HD1434-1721 and pGBT9-HD2226-3144 (filled boxes), used in LexA- and GAL4-based yeast two-hybrid cDNA library screens. The number of independent huntingtin interactors that each bait detected is given at the bottom.

Table 1. Summary of huntingtin yeast two-hybrid survey results

Bait clone	Huntingtin interactor	Identity	cDNA library	Accession no.; reference
pLexA-HD1-425Q62	HYP A	mouse FBP-11 WW domains,	pB42AD fetal brain	U40747; 26, AF049523, AF049524 AF049528
	HYP B	novel; WW domain	pB42AD fetal brain	AF049610, AF049103
	HYP D	MAGE-3	pB42AD fetal brain	U03735; 32
	HYP E	novel	pB42AD fetal brain	AF049611
	HYP F	26S proteasome subunit P31	pB42AD fetal brain	P48556; 28
	HYP A	mouse FBP-11 WW domains	pVP16 testis	U40747; 26 AF049523
	HYP B	novel; WW domain	pVP16 testis	AF049610
	HYP C	novel; WW domains	pVP16 testis	AF049525
	HYP G	hE2-25K; HIP-2	pGAD10 fetal brain	P27924; 29
	HYP H	novel; ankyrin repeats	pVP16 testis	AF049612
pGBT9-HD1-550 Q58	HYP I	symplekin	pVP16 testis	U88726; U49240; 30
	HYP J	mouse α -adaptin-C	pVP16 testis	B30111; 31 AF049527
	HYP K	novel	pVP16 testis	AF049613
	HYP L	novel	pVP16 testis	AF049614
	HYP M	novel	pVP16 testis	AF049615
	—		pGAD10 fetal brain; pVP16 testis	
pGBT9-HD530-1050	—		pB42AD fetal brain	
pLexA-HD1434-1721	—		pGAD10 fetal brain;	
pGBT9-HD1434-1721	—		pVP16 testis	
pGBT9-HD2226-3144	—		pGAD10 fetal brain; pVP16 testis	

Surprisingly, despite robust expression (data not shown) and screening of $>4 \times 10^6$ cDNAs, the internal and C-terminal baits comprising >70% of huntingtin yielded no interactor clones. This may indicate the lack of true interactor cDNAs in these libraries, failure of the bait polypeptides to assume native conformations or perhaps the inability of the bulk of huntingtin to participate in interactions with other proteins. In contrast, mutant N-terminal baits yielded 13 distinct HYPs, most represented by multiple independent cDNAs. Two, HYPA and HYPB, were also identified in screens of both the brain and testis cDNA libraries.

Description of huntingtin N-terminal interactors

To determine whether any of the HYPs encode previously described proteins that would implicate huntingtin in particular cellular processes, we searched DNA and protein databases (Table 1). Six of the HYPs represent known proteins associated with at least four different cellular pathways.

HYPA is the human homolog of mouse FBP-11, a formin-binding WW domain protein involved in spliceosome function (GenBank accession no. U40747) (26; M. Bedford, personal communication) that also exhibits extensive sequence identity to a predicted 84.7 kDa *Caenorhabditis elegans* WW domain protein, ZK1098.1 (accession no. P34600) and to two yeast proteins, *Schizosaccharomyces pombe* hypothetical protein Z98602 (accession no. S40923) and *Saccharomyces cerevisiae* splicing factor Prp40 (YKL012w; accession no. P33203) (27).

Two interactors, HYPF and HYPG, are involved in protein catabolism. HYPF is the 26S proteasome regulatory subunit P31, involved in the ATP-dependent degradation of ubiquitinated proteins (accession no. P48566) (28). HYPG, the UBC1 ubiquitin-conjugating enzyme hE2-25K (accession no. P27924) participates in the degradation of short-lived and misfolded proteins and has been reported previously as a huntingtin interactor (29).

HYPI and HYPJ are both proteins involved in the function of specialized membranes. HYPI, symplekin, is a protein at the cytoplasmic plaque face of certain tight junctions (accession no. U88726) (30) and is also found in the nucleoplasm. HYPJ, the human homolog of mouse α -adaptin-C (HYPJ) is a subunit of the plasma membrane adaptor assembly protein complex 2 in the coat surrounding the cytoplasmic face of coated vesicles (accession no. B30111) (31).

The other eight interactors have not been associated with particular functions, although one, HYPD, is the protein MAGE-3 (HYPD), a melanoma-associated antigen expressed in a variety of tumor cells (accession no. U03735) (32). Of the remaining seven, HYPE, HYPK, HYPL and HYPM display no significant DNA or amino acid homology to previously reported genes, while HYPB, HYPC and HYPH exhibit motifs that are found in a number of otherwise dissimilar proteins. Interestingly, HYPB and HYPC, like HYPA (FBP-11), possess related WW domains. HYPH displays homology with ankyrin repeats.

HYPA, HYPB and HYPC are members of a WW domain family

The fact that HYPA, HYPB and HYPC are related by shared motifs strongly suggests a role for a class of WW domain proteins in huntingtin's normal function and/or HD pathogenesis. We attempted to extend the cDNA sequences for these HYPs by

cDNA screening and expressed sequence tag (EST) database searching.

The overlapping HYPA brain and testis cDNAs contain tandem WW domain-coding sequences and together encode an open reading frame (ORF) of 423 amino acids (accession nos AF049523 and AF049524). The additional analyses revealed two alternate versions of the protein, one with a 42 amino acid insert upstream of the WW domains (accession no. AF049528) and another possessing an 18 residue insert downstream of the WW domains (Fas ligand-associated factor 1, accession no. U70667). The EST database did not add any 5' or 3' sequence, but confirmed multiple splice versions of HYPA, suggesting isoforms of the protein that may each have a different biological activity.

The independent brain and testis HYPB cDNA clones selected in our yeast two-hybrid screens encode portions of the same 127 amino acid ORF and each contains a single WW domain (accession no. AF049610). EST searching revealed a large number of cDNAs, from a wide variety of tissue sources, with sequence identity to HYPB, but none of these showed evidence of alternative splice forms.

We selected a single HYPC cDNA in the two-hybrid screen (accession no. AF049525). This clone contains an ORF of 143 residues, predicting a novel polypeptide with two tandemly arrayed, WW domain-related sequences. Database searches revealed three human ESTs but these did not extend the sequence of our HYPC cDNA. Interestingly, one of these ESTs appears to represent a splice variant lacking the sequences encoding both WW domains and the intervening spacer, increasing the complexity of isoforms expected from this family.

HYPA, HYPB and HYPC are different proteins that share related WW domains (Fig. 2). This motif, comprising 35–40 loosely conserved amino acids, is named for two invariant tryptophan residues and a conserved proline (22,26). There are at least two recognized classes of WW domains, typified by the representatives found in FBP-11 (U40747) and Yes kinase-associated protein 65 (YAP65, accession no. X80507), respectively (22). All three HYPA isoforms possess the same pair of domains which are perfectly conserved in mouse FBP-11. The tandem WW domains of HYPC exhibit strong similarity with those in HYPA, suggesting that these motifs may be functionally related. None of these WW domains is closely related to those of YAP65. FBP-11 and YAP65 bind distinct proline-rich sequences in their ligand proteins, supporting the idea that members of WW domain families serve different biological functions (26,33,34). Interestingly, the single WW domain in HYPB cannot be positioned unequivocally in either of the FBP-11 and YAP65 subclasses and may, therefore, represent an intermediate form or define a distinct subclass.

Expression of HYPA, HYPB and HYPC

To determine whether huntingtin's WW domain protein partners exhibit a broad expression pattern, like huntingtin itself, or a more restricted distribution consistent with the specific neuropathology of HD, we examined HYPA, HYPB and HYPC mRNA levels by northern blot analysis. The results, shown in Figure 3, reveal that HYPA, HYPB and HYPC mRNAs are expressed in adult and fetal brain and in a wide variety of peripheral tissues. An HYPA-specific probe (excluding the WW domain region) detects a major hybridizing band of ~4.2 kb in all tissues tested, and additional signals at ~3.4–3.7 and ~7.2 and 7.9 kb that are

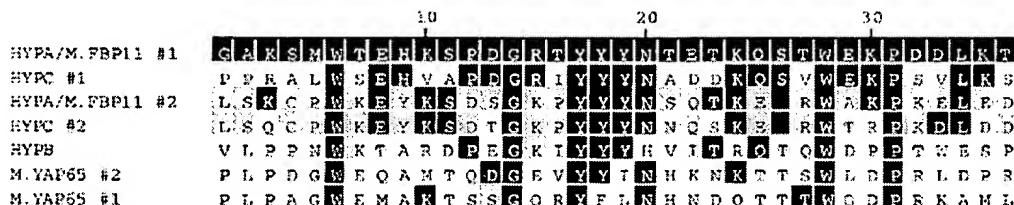


Figure 2. HYPA, HYPB and HYPC WW domains are family members. The individual WW domains in HYPA, HYPB, HYPC and mouse YAP65 (accession no. X80508) are aligned to illustrate amino acid sequence identity with the first (shaded black) and second (shaded gray) WW domains of HYPA/FBP-11 (accession no. U40747). The conserved tryptophans and proline residue, shared by all WW domain family members, are at positions 6, 28 and 31, respectively. Members of the HYPA/FBP-11 subclass typically bind proline-rich segments containing PPLP and possess three conserved internal tyrosine residues (positions 17–19) (26,34). Each module is shown with five flanking amino acids. The tandem motifs in HYPA/FBP-11 and HYPC are separated by a 15 amino acid spacer that is also highly conserved (not shown). The prolines substituted in the variant HYPB WW domain in the pB42AD-HYPBW/F prey construct (Fig. 4) are at positions 31 and 36. Multiple domains in a given protein are numbered from the N-terminus of the protein. Alignments with MegAlign software (Lasergene, DNASTAR).

consistent with the multiple different isoforms predicted by the EST database. HYPB, detected as a single mRNA of ~9.0 kb in all tissues tested, is expressed at levels comparable with HYPA, with the exception of fetal brain where it may be more abundant. In contrast, the HYPC mRNA level is lower (as predicted from the EST database), requiring a much longer exposure of these identical northern blot panels to detect a major broad signal at ~3.3–4.0 kb and minor bands at ~4.4–4.6 kb in most tissues. These results suggest that like HYPA, HYPC may comprise variably spliced mRNAs encoding alternate versions of this novel WW domain protein.

Interaction of HYPA and HYPB with huntingtin in the yeast two-hybrid system

To characterize precisely huntingtin's interaction with these WW domain proteins, we analyzed the two family members detected as multiple cDNAs in the yeast two-hybrid system. Figure 4 shows the activation of *LEU2* and *LacZ* reporters by specific interaction of HYPA and HYPB with the original bait, pLexA-HD1–425Q62, and the failure of each to interact with a non-huntingtin control. Varying huntingtin's glutamine segment from 62 to two residues diminished reporter gene activation, particularly with HYPA. To quantitate this trend, we measured β -galactosidase reaction product in a liquid culture assay (Table 2). The results for both HYPs demonstrate a progressive reduction in β -galactosidase activity as the number of glutamines in the huntingtin bait is decreased, suggesting that these partners bind preferentially to mutant huntingtin. They also reveal that, for

any given glutamine length, huntingtin interaction with HYPB yields a higher level of reporter gene activation than with HYPA.

To test the robustness of these interactions, we also reversed the bait-prey relationships and tested a different huntingtin N-terminal fragment. HYPA and HYPB DNA-binding domain fusion baits were assessed against preys with a longer (HD1–550) huntingtin segment containing either 23 or 62 glutamines. As shown in Figure 4, strong activation of reporter gene transcription was obtained despite the altered bait-prey configuration.

These results are consistent with the nature of the WW domain as a modular protein–protein interaction motif (35). Consequently, we also asked whether these WW domain proteins can undergo homo- or heterotypic interactions, expressing each as an activation domain and a DNA-binding domain fusion protein (Fig. 4). Singly, none of the HYPA and HYPB baits or preys produces a positive selection phenotype, indicating that neither HYPA nor HYPB has inherent DNA-binding or activation activity. However, co-expression of either an HYPA or an HYPB bait-prey fusion protein pair activates reporter gene transcription, indicating that these HYPs both possess self-association domains. These domains are apparently specific, as HYPA and HYPB do not interact with each other.

In these experiments, we also examined the self-association properties of huntingtin's N-terminus. Expression of pLexA-HD1–425Q2, Q23 or Q62 DNA-binding domain or pB42AD-HD1–550Q23 or Q62 activation domain fusion proteins, singly or together in any of the six possible bait-prey combinations, failed to yield interaction based on our stringent selection criteria (data not shown). Thus, regardless of polyglutamine length, these particular N-terminal fragments do not associate to form avid transcription complexes.

Table 2. Polyglutamine length dependence of the interaction with HYPA and HYPB

Bait clone	Prey clone	pB42AD (<i>n</i> = 6)	pB42AD-HYPA (<i>n</i> = 12)	PB42AD-HYPB (<i>n</i> = 14)
pLexA-HD1–425Q2	3.1 ± 1.3	55.3 ± 3.3 ^a	363.2 ± 19.0 ^a	
pLexA-HD1–425Q23	2.0 ± 1.0	118.1 ± 8.4 ^a	524.3 ± 19.3 ^a	
pLexA-HD1–425Q62	3.5 ± 1.6	220.8 ± 1.6 ^a	626.5 ± 34.4 ^a	

The results are given as β -galactosidase activity units for growth on selective media and are the mean values obtained in a given number of assays (*n*) for each bait-prey combination ± the standard error.

^aPairwise comparisons of the means for all groups were statistically significant ($P < 0.05$) as determined by the Student–Newman–Keuls method.

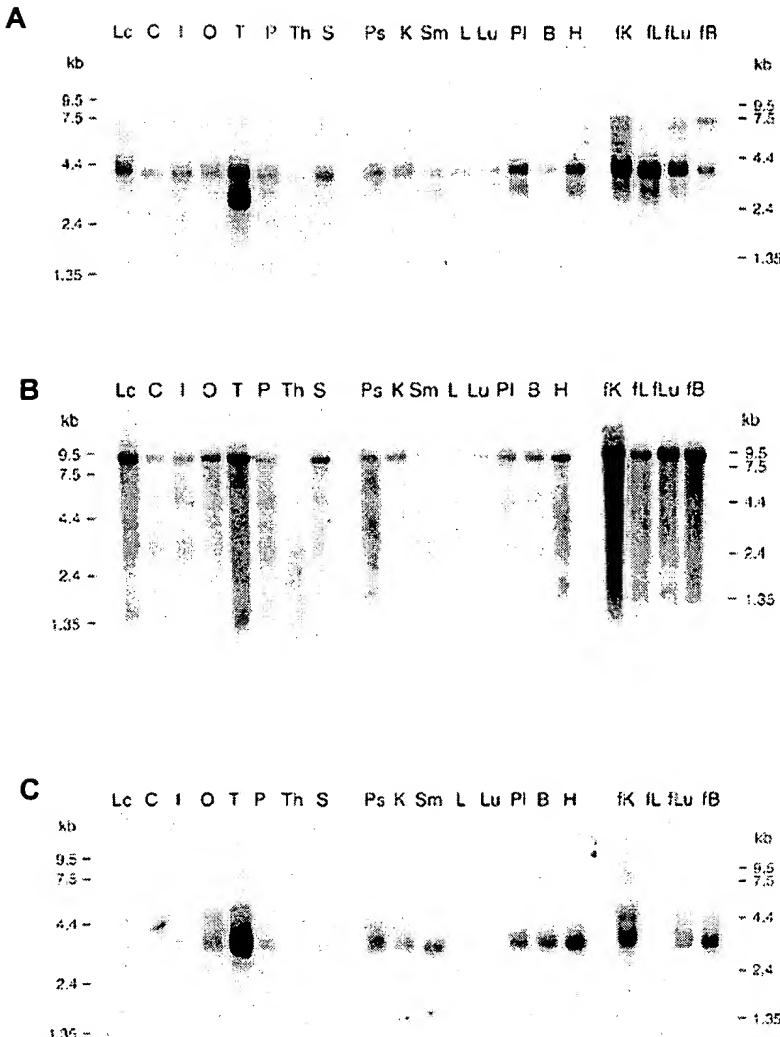


Figure 3. Northern blot analysis of HYPA, HYPB and HYPC. The pattern of HYPA- (A), HYPB- (B) and HYPC- (C) specific hybridization to northern blots of mRNA from adult and fetal human tissues is shown. Tissues are given above each lane: peripheral blood leukocytes (Lc), colon (C), ileum (I), ovary (O), testis (T), prostate (P), thymus (Th), spleen (S), pancreas (Ps), kidney (K), skeletal muscle (Sm), liver (L), lung (Lu), placenta (Pl), brain (B), heart (H), fetal kidney (fK), fetal liver (fL), fetal lung (fLu), fetal brain (fB). Thymus mRNA appears to be degraded. (C) was exposed four times longer than (A) and (B), and signals in all tissues except thymus could be detected by yet longer exposures. The molecular weight markers to the left refer to the adult blots and those to the right to the fetal blot.

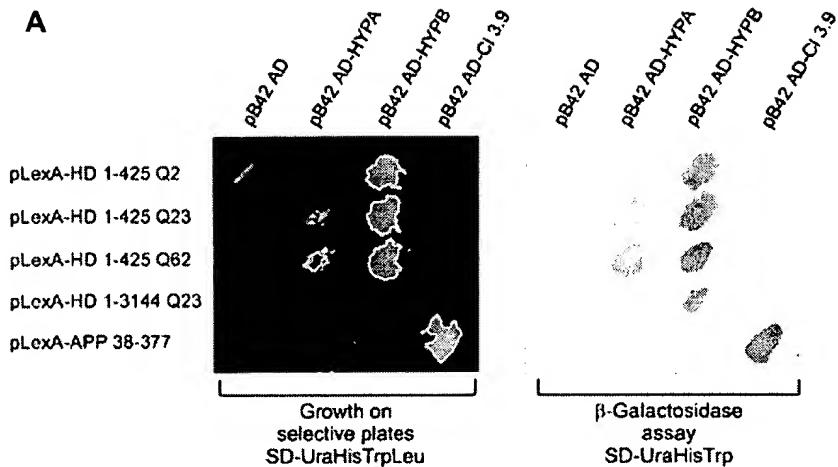
To determine whether huntingtin's WW domain protein interactors bind huntingtin via their related WW domains, we mutated HYPB's single WW domain motif, introducing codon changes that substitute phenylalanines for critical tryptophan residues (33). As shown in Figure 4, this mutant HYPB fusion protein fails to interact with N-terminal huntingtin baits but, interestingly, its ability to associate with wild-type HYPB is unimpaired. These observations reveal that identical structural features do not mediate HYPB homodimer formation and huntingtin binding. As HYPB's single WW domain mediates the association with huntingtin, the related tandem motifs in HYPA and HYPC almost certainly mediate the interactions of these

proteins with huntingtin's N-terminus. However, the presence of separate self-association domains in both HYPA and HYPB suggests that these WW domain proteins normally may form dimers *in vivo*.

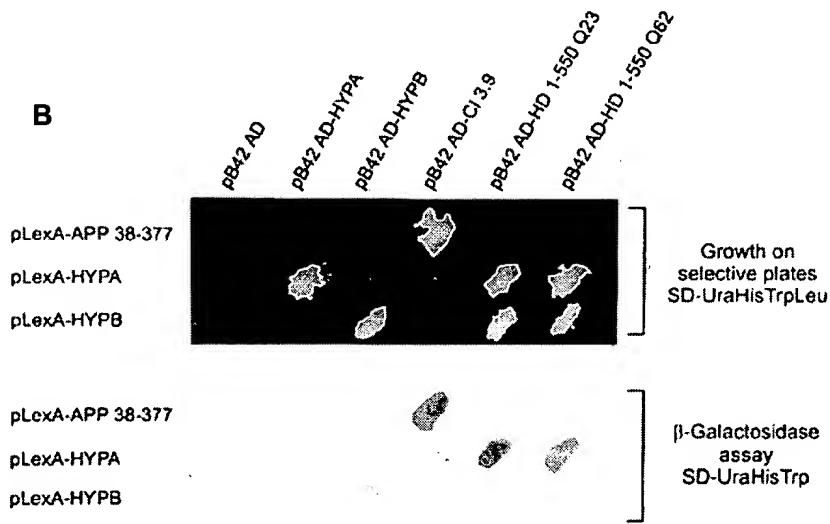
WW domain HYPs interact via huntingtin's N-terminal proline-rich segment

WW domains normally bind via short proline-rich segments in their protein ligands (26). FBP-11 and YAP65, representing separate classes of WW domain proteins, associate preferentially with ligands possessing PPLP and PY motifs, respectively

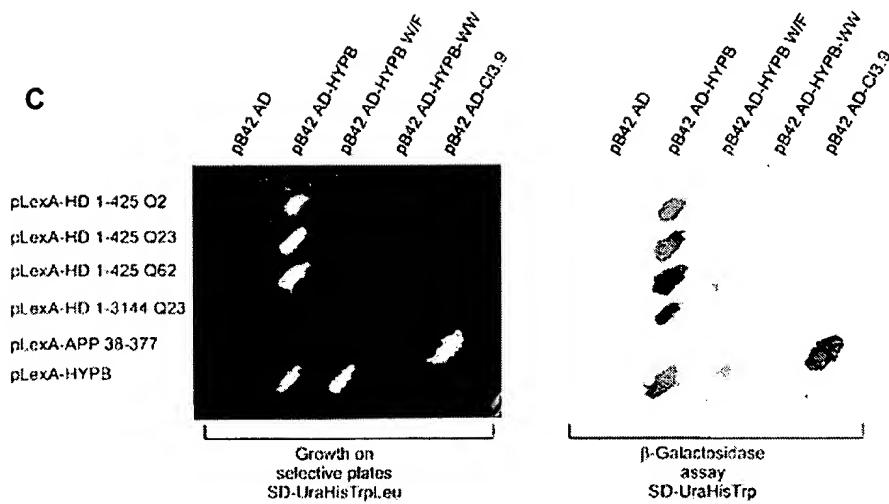
A



B



C



(33,34). Neither of these sequences is present in human huntingtin's N-terminal segment, although there is a proline-rich stretch following the glutamine tract. To delineate the portion of huntingtin's N-terminus responsible for WW domain binding, we used GST-HYP fusion proteins, including variants with distinct polyglutamine and proline-rich segments, to capture truncated huntingtin products expressed in COS7 cell extracts. HYPA, HYPB and HYPC each bind huntingtin with glutamine tracts of normal or disease-producing length (Fig. 5). To eliminate the possibility of binding to this tract, we shortened the polyglutamine to two residues. This did not eliminate binding, demonstrating that disease-associated lengths are not essential for huntingtin's interaction with its WW domain partners.

We next tested the adjacent proline-rich segment by removing the cognate sequences from the pcDNA3HD1-550Q2 construct. The modified polypeptide encoded by pcDNA3HD1-550Q2ΔP was detected by the anti-huntingtin antibody but was not captured by the GST-HYP fusion proteins, directly implicating huntingtin's proline-rich segment in these interactions. This target site shows specificity for a subset of WW domains, as it is captured by the tandem WW domain module of FBP-11 (identical to HYPA) but fails to bind to the analogous tandem WW domain module of mYAP65 fusion proteins (Fig. 5). Notably, while the human huntingtin N-terminus does not contain PPLP, the canonical FBP-11-binding sequence (34) is present in mouse and rat huntingtin homologs. Interestingly, another FBP-11/HYP ligand that lacks the PPLP motif, WBP-10 (34), has been shown to associate with the SH3 domains of abl, src and fyn, implying that huntingtin's proline-rich segment also may contain overlapping WW and SH3 protein-protein interaction domains.

In these experiments, we also detect huntingtin degradation products of variable intensity whose mobility shifts due to the variable polyglutamine stretch parallel the expected bands (Fig. 5). Notably, the GST-HYP fusion proteins also bind these smaller huntingtin fragments, indicating that they contain the proline-rich segment and implying that similar small fragments generated during HD pathogenesis (19) might be recognized *in vivo* by huntingtin's WW domain partners.

WW domain partners bind to huntingtin in HD lymphoblastoid cells

A physiological role for the WW domain partners in supporting the function of normal huntingtin or in triggering pathogenesis with mutant huntingtin would be most likely to result from an interaction with the native protein. As shown in Figure 6, all three partners, HYPA, HYPB and HYPC, bind full-length huntingtin in extracts of normal, HD heterozygote and HD homozygote lymphoblastoid cells. As expected, native huntingtin also interacts with isolated FBP-11 but not YAP65 WW domain modules.

Although huntingtin is captured efficiently by all three WW domain partners, the relative signal intensities of the huntingtin bands observed in several experiments suggests the relative order for preference of binding to be HYPB > HYPC > HYPA. Interestingly, the variation in huntingtin's polyglutamine length, ranging from 18 to 86 residues, did not strikingly affect binding to any of the WW domain partners, as both normal and mutant huntingtin were captured efficiently. However, these qualitative experiments do not exclude subtle differences in binding of normal and mutant huntingtin predicted by the yeast two-hybrid assays. Thus, an examination of the WW domain proteins in HD brain will be required to assess their potential involvement in HD pathogenesis.

DISCUSSION

Huntingtin's primary sequence provides no obvious clues to its physiological role or to the biochemical disruption by which the *HD* mutation leads to specific neuronal degeneration. Consequently, we have sought clues to these processes via huntingtin's interactions with other proteins. Our data indicate that the normal and mutant versions of huntingtin's N-terminus are capable of many diverse interactions that implicate huntingtin in a variety of cellular processes that are candidates for huntingtin's normal and/or abnormal function.

Huntingtin's N-terminus previously has been reported to associate in yeast two-hybrid assays with four proteins: HAPI (36), a novel protein implicated in intracellular protein trafficking (37,38); HIP1 (39,40), a human homolog of yeast cytoskeletal protein Sla2p; CBS, the enzyme cystathionine β -synthase (41); and HIP2, the hE2-25K ubiquitin-conjugating enzyme (HYPG) which is active in protein turnover (29). While our survey identified the latter protein, it also produced 12 new huntingtin-binding proteins, expanding considerably the scope of huntingtin's potential activities. Two of the new interactors, α -adaptin-C/HYPJ and the 26S proteasome regulatory subunit p31/HYPF, support previous proposals for huntingtin functioning in protein trafficking (37,38,42) and degradation (29). Two, HYPA (FBP-11) and HYPI (symplekin), suggest novel processes, mRNA splicing and tight junction function, respectively. However, for the majority of the interactors (HYPB, HYPC, HYPD, HYPE, HYPH, HYPK, HYPL and HYPM), more detailed characterization will be required to discover a functional role. Interestingly, HYPA (FBP-11) and HYPI (symplekin) are both detected in the cytoplasm as well as in the nucleus (L. Passani and M. MacDonald, unpublished data; 30), although their activities have been studied in one location. This suggests that these HYPs may have additional, as yet unrecognized activities. Interaction with such 'dual residence' partners implies that huntingtin's normal function may involve multiple intracellular addresses.

Figure 4. Yeast two-hybrid analyses of HYPA and HYPB. (A) The interaction of HYPA, HYPB and HYPC preys and huntingtin baits possessing different glutamine lengths. *LEU2* and *LacZ* reporter gene activation by HYPA (pB42AD-HYPA) and HYPB (pB42AD-HYPB) preys in combination with N-terminal (pLexA-HD1-425Q2 and Q62) and full-length huntingtin (pLexA-HD1-3144Q23) baits is shown by growth on selection medium and β -galactosidase assay, respectively. (B) The interaction of huntingtin preys with HYPA and HYPB baits and HYPA and HYPB self-association. *LEU2* and *LacZ* reporter gene activation by HYPA (pB42AD-HYPA), HYPB (pB42AD-HYPB) and huntingtin N-terminal (pB42AD-HD1-550Q23 and Q62) preys with HYPA (pLexA-HYPA) and HYPB (pLexA-HYPB) baits is assessed by growth on selection plates and β -galactosidase assay, respectively. (C) The interactions of huntingtin baits with variant HYPB WW domain preys. *LEU2* and *LacZ* reporter gene activation by wild-type (pB42AD-HYPB) or mutant (pB42AD-HYPB/F) WW domain HYPB preys, and the isolated HYPB WW domain (pB42AD-HYPB-WW) prey in combination with N-terminal (pLexA-HD1-425Q2-Q62) and full-length huntingtin (pLexA-HD1-3144Q23) baits is shown. The interaction of these preys with a HYPB (pLexA-HYPB) bait demonstrates self-association properties of HYPB. A non-huntingtin bait-prey control combination (pLexA-APP38-377 and pB42AD-Cl3.9) is shown in all panels.

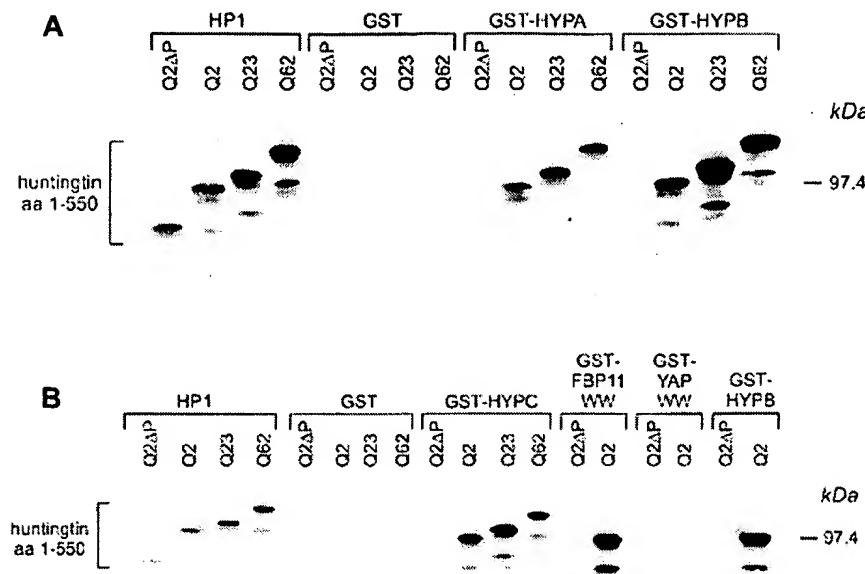


Figure 5. HYPA, HYPB and HYPC binding to huntingtin's N-terminus in COS7 cell extracts. The immunoblot in (A) shows the detection of huntingtin N-terminal fragments with anti-huntingtin antibody number 2166 in protein complexes isolated from transfected COS7 cell extracts by immobilized anti-huntingtin antibody HP1 (16,17), GST, GST-HYPA and GST-HYPB. (B) Results for HYPC (GST-HYPC) and tandem FBP-11 (GST-FBP-11 WW) and YAP65 (GST-YAP WW) WW domain module fusion proteins. The length of the glutamine tract, 2, 23 and 62 residues (Q2, Q23, Q62), or absence of the proline-rich stretch (Q2ΔP) in the huntingtin N-terminal fragments is given above each lane. The position of the molecular weight standard in kilodaltons (kDa) is indicated.

This is consistent with reports that while huntingtin is mainly a cytoplasmic protein, a small fraction may be found in the nucleus (43).

This abundance of N-terminal huntingtin interactors contrasts dramatically with the absence of cloned binding partners for the remainder of the protein. We uncovered no cDNA partners for huntingtin's highly conserved C-terminus or for two internal fragments that possess tandemly arrayed HEAT repeats (25), motifs that have been implicated in interactions between other proteins. Our results suggest that identification of binding partners for the C-terminal two-thirds of huntingtin will probably require alternative strategies to yeast two-hybrid screening.

Despite the lack of interactors for the bulk of the protein, huntingtin's N-terminal interactors provide a wealth of candidates that warrant further evaluation. Most obvious is the class of WW domain interactors represented by HYPA, HYPB and HYPC. WW domain proteins are currently the subjects of intense investigation as they have been found to play critical roles in a number of cellular processes (44). For example, YAP65 binds to the SH3 domain of the Yes non-receptor kinase, connecting it to intracellular signaling (45). NEDD4 is a ubiquitin-protein ligase implicated in reduced activity of the amiloride-sensitive epithelial sodium channel (46). Interestingly, mutations of the channel's PPPNY target sequence abolish binding of NEDD4's WW domains and thereby cause Liddle syndrome, an inherited form of hypertension (47). The WW domain of neural protein FE65 interacts with Mena, the mammalian homolog of *Drosophila* Enabled, a cytoskeletal protein involved in microfilament assembly (48). FE65 also interacts via its non-WW domain

region with the amyloid precursor protein (APP) central to the development of Alzheimer disease (49). WW domains are also found in proteins implicated in Ras or MAP kinase signaling pathways and in ligands that bind to transcription factors, RNA polymerase II, the dystrophin-associated *b*-dystroglycan receptor and formin (22,44). Notably, a class of WW domain proteins containing hect motifs interacts with atrophin-1, the defective protein in dentatorubral and pallidoluysian atrophy (DRPLA), another dominantly inherited CAG repeat expansion disorder (50).

Members of the HYPA/FBP-11 WW domain class form a distinct subclass that favors a ligand containing a PXXXPPLP target sequence (26,34). Several close variants of this target site are found in human huntingtin's proline-rich N-terminus, including PXXXPPXP motifs similar to a segment in the FBP-11 WW domain ligand WBP-10 (34). This particular target sequence is also recognized by the SH3 domains of abl, src and fyn (34), suggesting that huntingtin's N-terminus may also bind SH3 domain proteins. This possibility is supported both by a report of huntingtin's SH3-dependent association with epidermal growth factor (EGF) receptor signaling complexes (51) and by our isolation of SH3P12, a mouse SH3 domain peptide ligand (52), using huntingtin N-terminal baits to screen a mouse embryo interaction cDNA library (C. Dompe and M. MacDonald, unpublished data).

The HYPs also present a number of new entrees into the exploration of HD pathogenesis, including both previously suggested pathways, such as vesicle transport and huntingtin degradation, and new directions involving membrane, nuclear

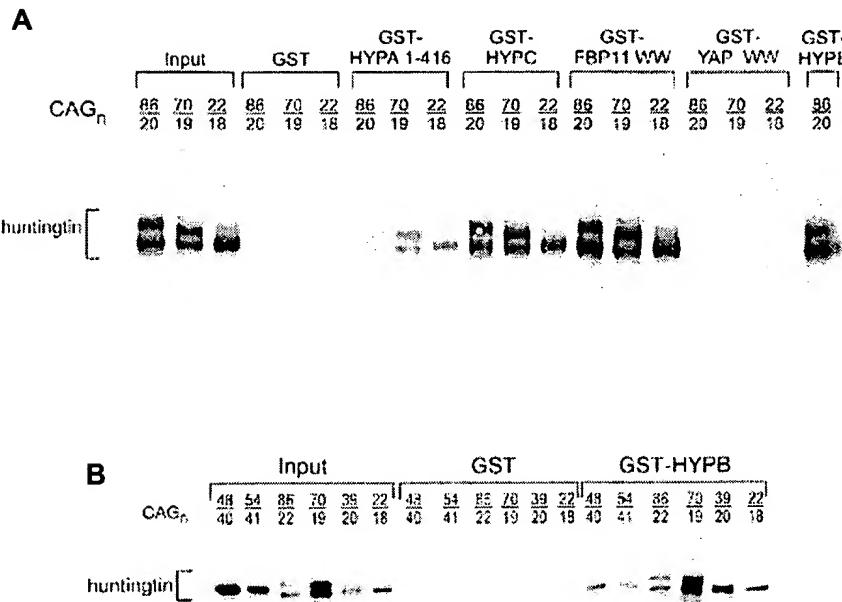


Figure 6. HYPA, HYPB and HYPC binding to huntingtin in normal and HD patient lymphoblastoid cell extracts. The immunoblots in (A) and (B) show detection by anti-huntingtin antibody of full-length endogenous normal (~350 kDa) and mutant (>350 kDa) huntingtin with the different polyglutamine lengths encoded by the polymorphic *HD* CAG repeat (CAG_n) indicated above each lane. Proteins from lymphoblastoid extracts (Input) were bound specifically by immobilized GST, GST-HYPA 1-416, GST-HYPC, GST-FBP-11 WW and GST-YAP65 (A) or from GST-HYPB (B). A single lane of GST-HYPB-bound proteins is shown in (A) for comparison. Normal individuals are (CAG 22/18), HD heterozygotes (CAG 39/20, 70/19, 86/20, 86/22) and HD homozygotes (CAG 48/40, 54/41).

and cytoskeletal functions. Although none of the HYPs exhibits a striking difference in binding to normal and mutant huntingtin, subtle differences, consistent with the late onset and long duration of the disorder, were evident in the yeast two-hybrid assays. Several scenarios are possible with any huntingtin interactor: (i) a change in binding could, over time, cause the gradual loss of a huntingtin activity or an activity of the interactor, assuming that these are crucial for neuronal cell survival; (ii) an aberrant association could cause an increase in activity of either huntingtin or the interactor with potential toxic consequences; and (iii) an altered interaction might have an indirect consequence, such as promoting the formation of insoluble aggregates that are capable of killing target neurons independently of any coincident effect on huntingtin or interactor activity. The diversity of the proteins that interact with huntingtin's N-terminus broadens the options for investigation of the selective neuronal cell loss in HD under any of these three hypotheses. Moreover, the discovery of a class of WW domain interactors suggests a number of specific functions, such as pre-mRNA splicing, to be explored in HD brain and model systems.

MATERIALS AND METHODS

HD expression constructs

Huntingtin yeast two-hybrid, *Escherichia coli* and mammalian clone constructs were generated using restriction fragments and PCR products from pBS-HD1-3144Q23 and pBS-HD1-3144Q62. These encode full-length huntingtin with 23 and 62 glutamines, respectively, and were assembled in pBluescriptII (Stratagene) from

partial *HD* cDNAs (3; accession no. L12392, 343–10 200 bp) and a 5' exon 1 *HD* genomic fragment (196–343 bp). Huntingtin expression constructs were created in pLexA (pJG4-5; 53,54), pGBT9 (accession no. U07646, Clontech), pB42AD (pJG4-5; 53,54), pGEX-2TK (Pharmacia) and pcDNA3FLAG, a modified pcDNA3 (Invitrogen) vector kindly provided by Dr V. Ramesh. In all cases, the clones were validated by DNA sequencing (55) and by SDS-PAGE gel analysis of protein products synthesized in a coupled *in vitro* transcription/translation reaction (TNT; Amersham). Huntingtin bait clones pLexA-HD1-425Q2, pLexA-HD1-425Q23 and pLexA-HD1-425Q62 encode residues 1–425 (T388P) with two, 23 and 62 glutamines, respectively, while baits pGBT9-HD1-550Q23 and pGBT9-HD1-550Q58 and preys pB42AD-HD1-550Q23 and pB42AD-HD1-550Q62 encode huntingtin amino acids 1–550 with either 23, 58 or 62 glutamines. Huntingtin bait pLexA-HD1-3144Q23 encodes full-length huntingtin with 23 glutamines. Internal bait pGBT9-HD530–1050 expresses huntingtin amino acids 530–1050, whereas baits pLexA-HD1434–1721 and pGBT9-HD1434 encode huntingtin residues 1434–1721. The C-terminal huntingtin bait pGBT9-HD2226–3144 comprises amino acids 2226–3144. Mammalian expression constructs pcDNA3-FLAGHD1-550Q23 and pcDNA3-FLAGHD1-550Q62 produce 5' FLAG-tagged huntingtin N-terminal fusion proteins (residues 1–550) with 23 and 62 glutamines, respectively. pcDNA3FLAG-HD1-550Q2 and pcDNA3FLAG-HD1-550Q2ΔP express variant N-terminal fragments with two glutamines, plus or minus the adjacent proline-rich segment (436–546 bp). Note that the number of consecutive glutamines in

each construct is greater by two than the number of CAG repeats, due to the CAACAG codons that follow the CAG tract (3).

HYP expression clones

Baits pLexA-HYPA and pLexA-HYPB were generated using the entire HYPA and HYPB cDNA insert, respectively. pB42AD-HYPBW/F encodes a HYPB fusion protein with tryptophan to phenylalanine substitutions (W31F; W36F) created by PCR-based site-specific mutagenesis. pB42AD-HYPB-WW encodes HYPB's single WW domain (residues 1–57). The *E.coli* expression constructs encoding GST–HYPA, GST–HYPB and GST–HYPc fusion proteins were generated from HYP cDNAs. GST–HYPA1–146 expresses HYPA residues 1–146. pGEX-2TK-FBP-11 WW and pGEX-2TK-mYAP65 WW encode GST–FBP-11 and GST–YAP65 isolated tandem WW domain fusion proteins, respectively (34), and were kindly provided by Drs M. Bedford and P. Leder.

Control plasmids

The pLexA-APP38–377 and pB42ADCl 3.9 bait–prey combination, encoding a LexA–APP fusion protein and a positive yeast two-hybrid interactor, respectively, were a gift of Drs S. Guenette and R. Tanzi. Other non-huntingtin bait controls were pRFHM1 (23) and pLexA-TBP1–104Q38, which produces a LexA–TATA-binding protein (TBP) fusion protein with 38 glutamine residues. The non-huntingtin control pGBT9 bait–prey plasmid combinations for the GAL4 system were pVA3 and pTD1 (Clontech).

Yeast two-hybrid screens

General yeast two-hybrid methods have been described previously (57). Yeast hosts were EGY48 (*MATα his3 leu2::3Lexop-LEU2 ura3 trp1 LYS2*) (22,47,48) (*LexA*) and MAV103 (*MATα ura3-52 leu2-3 URAS::SPAL10-URA3-112 trp1-901 his3Δ200 ade2-101 GAL4 Δ gal80 Δ can1^R cyh2^R LYS2::GAL1-HIS3 GAL1-lacZ*) (56) (*GAL4*). Screens of a human fetal brain frontal cortex cDNA library in pB42AD (provided by Drs D. Krainc and R. Brent) (*LexA*), a human fetal brain library in pGAD10 (Clontech) or a human testis library in pVP16 (Drs K. Tashiro and I. Verma) (*GAL4*) were performed as described (23,53,54,56). Primary transformants were selected by growth for 3–5 days at 30°C on medium lacking uracil, histidine, tryptophan and leucine in the presence of galactose (*LexA*) or, for the other system, media with 75 mM 3-amino-triazole and glucose in the absence of histidine, tryptophan and leucine, followed by growth on glucose plates lacking uracil, tryptophan and leucine (*GAL4*). Primary transformants were then tested for β-galactosidase activity using a filter assay for the conversion of Xgal for 1 (*LexA*) or 6–8 (*GAL4*) h. cDNA inserts in doubly (*Lex*) or triply (*GAL4*) positive transformants were obtained by PCR amplification and characterized and grouped into classes by restriction enzyme and Southern blot analyses. Unique cDNA plasmids were shuttled into *E.coli*, purified and sequenced by dideoxy chain termination (55). The interaction properties of the longest purified interactor cDNA in each class were tested for activation of reporter genes using huntingtin (pLexA-HD1–425Q2, Q23 and Q62; pGBT9HD1–550Q23 and Q62) and non-huntingtin (pRFHM, pEG202-TBP1–104Q38, pVA3) baits. β-Galactosidase reaction product was measured as described (Clontech Matchmaker) using the formula: β-galactosidase units = 1000 × (OD₄₂₀/t × V × OD₆₀₀)

where t = incubation time (minutes) and V = volume (ml) of culture. Statistical analysis software was SigmaStat (Jandel Scientific).

Protein extracts, *in vitro* binding, immunoprecipitation and immunoblot analyses

GST fusion proteins were expressed from pGEX-2TK constructs in BL21(DE3)pLysE by induction with isopropyl-β-D-thiogalactopyranoside (IPTG): GST, GST–HYPB and GST–FBP-11 WW with 0.3 mM IPTG for 2 h at 37°C; GST–HYPA and GST–mYAP65 with 0.03 mM IPTG for 30 min at 37°C; and GST–HYPc with 0.03 mM IPTG for 5–6 h at room temperature. The GST fusion proteins in cleared *E.coli* sonicates were bound to glutathione–Sepharose 4B beads (Pharmacia) by incubation in binding buffer (150 mM NaCl, 50 mM Tris–HCl, pH 7.5, 0.5% NP-40, 2.5 mM MgCl₂, 2.5 mM KCl, 1.5 mM CaCl₂) for 30 min at 4°C. Mammalian cell extracts (at ~10 mg/ml) were prepared as described (16,17) from immortalized lymphoblastoid cells established previously from normal and HD individuals genotyped for the *HD* CAG repeat (3,16) and from COS7 cells 48 h after transfection. Proteins were captured by GST fusions by incubating lymphoblastoid and COS7 cell extracts (~500 µg) with ~1 µg of washed GST fusion protein–Sepharose beads in 300 µl of binding buffer for 1 h at 4°C. Washed bound beads (binding buffer containing 3% NP-40) were boiled in SDS-PAGE sample buffer to release captured proteins. Immunoprecipitation of full-length and truncated huntingtin was accomplished using HF-1, a polyclonal anti-huntingtin antisera as described previously (16,17). Protein complexes from GST fusion protein and immunoprecipitation assays were displayed by electrophoresis on 5 or 7% SDS-PAGE gels and transferred to nitrocellulose by electroblotting. Immunoblots were probed with the anti-huntingtin antibody mAb number 2166 (Chemicon) using enhanced chemiluminescence detection (ECL; Amersham). Protein concentration was determined by modified Bradford assay (Bio-Rad).

Northern analysis

Multiple human adult and fetal tissue northern blots (~1 µg of mRNA per lane) (Clontech) were hybridized with ³²P-labeled HYPA-, HYPB- and HYPc-specific cDNA probes corresponding to bp 834–1253 (accession no. AF049523), bp 164–883 (accession no. AF049610) and bp 1–197 (accession no. AF049525), respectively. Probes were labeled with [³²P]dATP using random oligonucleotide priming as described previously (58). Blots were exposed to X-ray film for 16 (HYPA, HYPB) and 64 (HYPc) h.

DNA, protein and EST database searches were performed using BLAST 2.0 programs (59) including Basic BLAST on the NCBI server.

Accession numbers

HYPA AF049523, AF049524, AF049528; HYPB AF049610, AF049103; HYPc AF049525; HYPE AF049611; HYPH AF049612; HYPI AF049526; HYPJ AF049527; HYPK AF049613; HYPL AF049614; HYPM AF049615.

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